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Fish Oil Increases the Duodenal Flow of Long Chain Polyunsaturated Fatty Acids and *trans*-11 18:1 and Decreases 18:0 in Steers via Changes in the Rumen Bacterial Community[1,2]

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Abstract

Ruminant fat is rich in SFA, partly due to the biohydrogenation of dietary PUFA to SFA in the rumen. This process can be inhibited by the dietary inclusion of fish oil. The only bacteria isolated from the rumen capable of converting PUFA to SFA are closely related to *Clostridium proteoclasticum*. The aim of this study was to investigate if a correlation could be found in vivo between dietary fish oil inclusions and the composition of the ruminal bacterial community and specifically of *C. proteoclasticum*. Six Hereford × Friesian steers, prepared with ruminal and duodenal cannulae, received grass silage plus 1 of 3 concentrates resulting in total dietary fish oil contents of 0, 1, or 3% of dry matter. A dual flow marker technique was employed to estimate the relative flow of fatty acids. Steers fed the 3% fish oil diet had 100% increases in *trans* 18:1 flow, whereas 18:0 flow declined to 39% of steers fed the control diet. 16S ribosomal RNA-based denaturing gradient gel electrophoresis profiles obtained from ruminal digesta showed major changes in the bacterial community within steers fed the 3% fish oil diet. Quantitative PCR indicated only a weak relation between numbers of *C. proteoclasticum* and 18:0 flow between treatments and in individual steers ($P < 0.05$, but the percentage variance accounted for only 22.8) and did not provide unambiguous evidence that numbers of *C. proteoclasticum* in the rumen dictate the ratios of SFA:PUFA available for absorption by the animal. Understanding which microbes biohydrogenate PUFA in the rumen is key to developing novel strategies to improve the quality of ruminant products. J. Nutr. 138: 889–896, 2008.

Introduction

Recent nutritional advice has emphasized the need to decrease intake of SFA and increase intakes of beneficial PUFA, in particular α-linolenic acid [18:3(n-3)] and the longer chain (n-3) PUFA [20:5(n-3) and 22:6(n-3)], in the diet (1). Ruminant products make an important contribution to the human diet but have caused concern due to their enriched SFA content (2). This is largely due to microbial biohydrogenation of dietary unsaturated fatty acids in the rumen, although some intermediates of biohydrogenation such as conjugated linolenic acid (*cis*-9, *trans*-11 CLA)[7] and *trans*-11 18:1 could be important in human health (3). Previous studies have shown that including fish oil in the diet of beef cattle resulted in increased long chain (longer than 20 C) PUFA (LCPUFA) in muscle resulting in a lower (n-6):(n-3) fatty acid ratio (4). Fish oil has also been shown to interrupt the complete biohydrogenation of C18 PUFA, resulting in increased production of *trans*-11 18:1 (5–7), the precursor for CLA (*cis*-9, *trans*-11) in the mammary gland (8).

The bacteria involved in the different steps of the biohydrogenation pathway have been categorized as Group A and B (9): group A bacteria hydrogenate 18:2(n-6) and 18:3(n-3) to *trans*-11 18:1; in contrast, group B bacteria convert the same fatty acids to 18:0. The only Group B bacteria identified for many years was '*Fusocillus*' spp. (10,11). Modern phylogenetic analysis of recent isolates has now shown that 18:0-forming bacteria, like the most active Group A bacteria, are part of the *Butyrivibrio fibrisolvens* group, an ill-defined taxon that includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* and the species *Clostridium proteoclasticum* (12,13). Group B bacteria (18:0 producers) form a tight grouping in which strains cluster together close to *C. proteoclasticum* (14,15). For this reason, in this article, the 18:0 producers are described as *C. proteoclasticum*.

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[6] These authors contributed equally to this study.
[7] Abbreviations used: CLA, conjugated linoleic acid; DGGE, denaturing gradient gel electrophoresis; DM, dry matter; LCPUFA, long chain PUFA; NDF, neutral detergent fiber; OM, organic matter, QPCR, quantitative PCR; rRNA, ribosomal RNA; VFA, volatile fatty acid.
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Advances in molecular microbial technology based on 16S ribosomal RNA (rRNA) genes mean that we are now able to quantify these bacterial groups using quantitative PCR (QPCR) and to investigate total eubacterial and *Butyrivibrio*-specific population changes using denaturing gradient gel electrophoresis (DGGE) (16). One of the major advantages of these molecular methodologies is the avoidance of an often laborious cultivation step that is frequently error-prone due to media selectivity and the suspected existence of noncultivable bacteria.

The aims of this study were to assess the involvement of *C. proteoclasticum*, other *Butyrivibrio*-related spp. and eubacteria in general in the biohydrogenation pathways operating in the rumen.

Materials and Methods

Animals, diets, and experimental design. The experiment was conducted under the authorities of the UK Animal (Scientific Procedures) Act (1986). Six Hereford × Friesian steers (609 ± 6.9 kg) prepared with ruminal cannulae and simple T-piece cannulae in the proximal duodenum [immediately post-pylorus and pre-common bile duct; (17)] were offered grass silage plus 1 of 3 concentrates: F0, F1, and F3 that provided increasing amounts of fish oil: 0, 23, and 69 g · kg^{-1} dry matter (DM), respectively (0, 1, and 3% of DM intake, respectively). The grass silage was prepared from first-cut perennial ryegrass (*Lolium perenne*) in May 2002. Salmon (*Salmo salar*) oil was purchased from the International Fish Meal and Oil Manufacturers Association. To maintain an iso-lipid nature of the diets, the addition of fish oil was balanced by the removal of Megalac (Volac; **Table 1**). The total daily allowance was set at 14 g DM · kg^{-1} live weight to ensure complete daily consumption with a forage: concentrate ratio of 60:40 (DM basis). Steers were housed in individual pens and transferred to stalls for each measurement period. The building was well ventilated and steers had free access to fresh water and mineral blocks (Baby Red Rockies, Tithebarn; composed of 380 g · kg^{-1} Na, 5000 mg · kg^{-1} Mg, 1500 mg · kg^{-1} Fe, 300 mg · kg^{-1} Cu, 300 mg · kg^{-1} Zn, 200 mg · kg^{-1} Mn, 150 mg · kg^{-1} I, 50 mg · kg^{-1} Co, and 10 mg · g^{-1} Se).

The experimental design consisted of a replicated 3 × 3 Latin Square with 2 steers per treatment. Each 21-d period consisted of 17 d of adaptation to the experimental diets and 4 d for sample collection. Steers received their daily allocations in 2 equal meals at 0900 and 1600. Digesta flow at the duodenum was estimated using a dual-phase marker technique with ytterbium acetate and chromium EDTA as the particulate and liquid phase markers, respectively (18). Ytterbium acetate (375 mg · d^{-1} Yb) and chromium EDTA (2401 mg · d^{-1} Cr) were infused via separate lines intraruminally at a rate of 28 mL · h^{-1} commencing on d 8. On d 18 and 19, 400 mL of duodenal digesta were collected manually every 3 h over a 24-h period, bulked, and stored at 4°C for further fractionation (see below). Samples of strained ruminal fluid (1 L) were taken 2 h post-feeding, for microbial analysis on d 21 and 10 mL strained ruminal fluid were taken every 2 h over a 12-h period (0830 to 2030) to measure pH, ammonia-nitrogen (ammonia-N), and volatile fatty acids (VFA) concentration in the rumen.

TABLE 1 Formulation of the concentrate component of experimental diets

Ingredients	Diet		
	F0	F1	F3
	g · kg^{-1} DM		
Barley	535	537	546
Molasses sugar beet pulp	200	200	200
Molasses	50	50	50
Soya bean meal	110	110	110
Megalac	80	55	0
Fish oil (*Salmo salar*)	0	23	69
Mineral premix[1]	25	25	25
Total	1000	1000	1000

[1] Mineral premix was obtained from Baby Red Rockies, Tithebarn Ltd. (Winsford, Cheshire, UK) and was composed of 380 g kg^{-1} Na, 5,000 mg kg^{-1} Mg, 1,500 mg kg^{-1} Fe, 300 mg kg^{-1} Cu, 300 mg kg^{-1} Zn, 200 mg kg^{-1} Mn, 150 mg kg^{-1} I, 50 mg kg^{-1} Co, and 10 mg kg^{-1} Se.

Sample preparation and chemical analysis. Accumulated samples of daily duodenal digesta were thoroughly mixed and a whole and centrifuged fraction produced as described by Lee et al. (19). Separate samples of silage and concentrate were taken daily (~500 g) during the sampling periods and pooled subsamples were freeze-dried, ground, and retained at −20°C for chemical analysis (19). The microbial fraction of ruminal fluid was obtained as described by Lee et al. (20) and freeze-dried and ground before molecular microbial analysis ensued. The fatty acids in the silage and concentrate were measured using a 1-step extraction-transesterification procedure (21). Digesta fatty acids were obtained by direct hydrolysis at 60°C, with added internal standard (100 μL 21:0 methyl ester, 15 g · L^{-1} $CHCl_3$), in 5 mol · L^{-1} KOH in aqueous methanol. Potassium carboxylates were converted into fatty acids by the addition of 5 mol · L^{-1} H_2SO_4 and methylated using 5% HCl in methanol at 50°C (22). Samples were analyzed by GC on a CP-Select chemically bonded for FAME column (100-m × 0.25-mm i.d.; Varian), split injection 30:1, helium carrier gas, and a temperature gradient program according to Lee et al. (6). Peaks were identified from external standards (ME61, Larodan Fine Chemicals; S37, Supelco; CLA, Matreya) and quantified using the internal standard (21:0) using the Varian star 6.4.1 software.

DNA extraction from rumen microbial samples. Genomic DNA was extracted from rumen microbial samples (10 mg DM) using the BIO101 FastDNA SPIN kit for soil (Qbiogene) in conjunction with a FastPrep cell disrupter instrument (Bio101, ThermoSavant, Qbiogene) according to the manufacturer's instructions, except that the samples were processed for 3 × 30 s at speed 6.0 in the FastPrep instrument. The integrity of the DNA was verified by agarose gel electrophoresis.

PCR-DGGE analysis of the total eubacterial population and the Butyrivibrio group. Amplification of the V6-V8 region of the 16S rRNA gene was carried out with the primer pair F968GC (5′-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and R1401 (5′-CGG TGT GTA CAA GAC CC-3′) (23,24), and F968GC and B fib (5′-TTC GGG CAT TYC CRA CT-3′) for total eubacterial and *Butyrivibrio* group-specific PCR, respectively. The 16S rRNA-targeted *Butyrivibrio* spp. specific reverse primer was based on a *B. fibrisolvens* probe published by Klieve et al. (25), but it was modified slightly so that it would amplify all members of the *Butyrivibrio* group while still fitting the criteria required for PCR-DGGE. In brief, we checked specificity in silico using an alignment of rumen bacterial sequences previously generated (26) and using the Probe Match tool in the Ribosomal Database Project-II release 9.42 (27). Once specificity for the *Butyrivibrio* group was determined, coverage of this group was investigated by aligning all *Butyrivibrio* group 16S rRNA gene sequences deposited in GenBank and EMBL databases using the program ClustalW (28). Based on this analysis, 2 bases were changed to include degeneracy to amplify all members of the *Butyrivibrio* group while maintaining specificity. Further specificity of the newly developed primer was confirmed by PCR of many pure cultures. Various Mg concentrations and annealing temperatures were investigated so that maximum specificity could be obtained while ensuring sensitivity at the same time. A Mg concentration of 3 mmol · L^{-1} was required to ensure sensitivity and an annealing temperature of 58°C was required to obtain specificity (data not shown). Specificity was then checked using DGGE analysis of ruminal digesta samples obtained from 2 ruminally cannulated Holstein-Friesian nonlactating dairy cows fed grass silage as described below. Dominant DGGE bands were excised and sequenced revealing that this PCR-DGGE amplified both cultivable and as yet uncultivated members of the *Butyrivibrio* group. Of 15 clones obtained from 5 dominant bands, 6 had 91% identity to *Eubacterium cylindroides*

L346616, so it should also be noted that this PCR-DGGE may amplify *Eubacterium* spp. The reverse B fib primer does have one and sometimes 2 mismatches in the middle of the primer for sequences deposited for cultivable *Eubacterium* spp. and in the presence of eubacterial 16S rRNA having a 100% match with this primer, amplification of *Eubacterium* spp. should be minimal.

All PCR amplifications were performed using a 2720 thermal cycler (Applied Biosystems) in 50-μL volumes containing 1× PCR buffer (20 mmol · L^{-1} Tris HCl, pH 8.4, 50 mmol · L^{-1} KCl), 3 mmol · L^{-1} $MgCl_2$, 200 μmol/L deoxyribonucleotide triphosphate mix, 500 mmol · L^{-1} each primer, and 1.25 U of iTaq DNA polymerase (Bio-Rad Laboratories) with ~100 ng of DNA template. Amplification conditions were: an initial denaturation of 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 56°C (total eubacteria) or 58°C (*Butyrivibrio* group) for 30 s and 72°C for 1 min, and then a final extension of 72°C for 5 min. Amplification of products was verified by agarose gel electrophoresis.

Amplicons were loaded onto 6% polyacrylamide gels with a 35–60% (total eubacteria) or a 35–65% (*Butyrivibrio* group) denaturing gradient [100% denaturant consisting of 40% (v:v) deionized formamide and 7 mol/L urea] and electrophoresis performed in a D-Code system (Bio-Rad Laboratories) as described previously (29). Gels were then stained with $AgNO_3$ (30). Gels were scanned using a GS-710 calibrated imaging densitometer (Bio-Rad Laboratories) and the saved image imported into the software package Fingerprinting (Bio-Rad Laboratories) for analysis. DGGE banding patterns were analyzed based on the presence and absence of the bands and resultant binary matrices were translated into distance matrices using the Dice similarity coefficient, with a position tolerance of 0.5% and optimization parameter of 1%. Finally, clusters were constructed using the method of unweighted pair group method with arithmetic mean analysis. The binary data generated were used to calculate band number and the Shannon-Weiner diversity index (24,31) using the Fingerprint Analysis with Missing Data software (32).

QPCR analysis. Total eubacterial amplification was carried out on ruminal digesta samples in a final volume of 25 μL containing 12.5 μL SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 250 nmol · L^{-1} each of EubF1 5′-GTG STG CAY GGY TGT CGT CA-3′ and Eub R1 5′-GAG GAA GGT GKG GAY GAC GT-3′ (33), and 2 μL of a 1:100 dilution of extracted genomic DNA. The thermal cycling program was 30 cycles of 94°C for 30 s and 61°C for 30 s with an initial cycle of 94°C for 5 min. After PCR, a dissociation curve (melting curve) was constructed in the range of 55°C to 95°C. All samples were run in triplicate. A bacterial standard was prepared with equal amounts of genomic DNA from 8 different pure cultures of bacteria: *Clostridium aminophilum* (ATCC 49906), *Peptostreptococcus anaerobius* (ATCC 27337), *Prevotella ruminicola* (ATCC 19189), *Fibrobacter succinogenes* (ATCC 19169), *B. fibrisolvens* [JW11; Rowett Research Institute (RRI)], *Ruminococcus albus* (SY3; RRI), *Selenomonas ruminantium* (Z108; RRI), and *Streptococcus bovis* (ES1; RRI).

QPCR analysis of 18:0-producing bacteria was conducted according to the method by Paillard et al. (34) on rumen-derived samples. Dilutions of purified genomic DNA from the control strain *Clostridium proteoclasticum* P-18 (RRI) were used to construct specific calibration curves. All samples were run in triplicate. Amplification was carried out in a final volume of 25 μL containing 12.5 μL of JumpStart Taq ReadyMix (Bio-Rad Laboratories), 400 and 800 nmol · L^{-1} of forward (SA-FW; 5′-TCC GGT GGT ATG AGA TGG GC) and reverse primers (SA-RV; 5′-GTC GCT GCA TCA GAG TTT CCT-3′), respectively, 250 nmol · L^{-1} of molecular beacon (5′-6 FAM-CCG CTT GGC CGT CCG ACC TCT CAG TCC GAG CGG-DABCYL-3′), and 2 μL of a 1:10 dilution of extracted genomic DNA. The thermal cycling program was 40 cycles of 30 s at 95°C, 1 min at 55°C, and 30 s at 72°C with an initial cycle of 95°C for 10 min. Fluorescence data were collected at the end of the hybridization step at excitation and emission wavelengths of 490 and 530 nm, respectively.

All QPCR were performed using an iCycler iQ thermal cycler (Bio-Rad Laboratories) and results were analyzed using the iCycler iQ detection system software (Bio-Rad Laboratories).

Calculations and statistical analysis. Digesta flows were estimated after mathematical reconstitution of true digesta as described by Faichney (18). Biohydrogenation of PUFA and LCPUFA was assessed as the difference between daily intake and duodenal flow (g · d^{-1}) as a proportion of daily intake. Data for intakes and flows of nutrients [organic matter (OM), total N, neutral detergent fiber (NDF), and fatty acids], for rumen fermentation characteristics (pH, ammonia-N, VFA), and for eubacterial QPCR quantification were subjected to Restricted maximum likelihood analysis using GenStat [release 9.1, (35)]. The model included carryover and diet (treatment) as the fixed effect, and steer, period, and their interactions as the random effect. Treatment effects were further partitioned using a polynomial contrast to evaluate the significance of linear and quadratic components of the response to treatments. The level treated as not significant was $P > 0.05$, but trends were also expressed ($P < 0.1$). For band number and Shannon-Weiner diversity index data, difference was also inferred by using a Student's *t* test (32,35). For the correlation between the flow of 18:0 and the DNA concentration of 18:0-producing bacteria, bootstrap analysis (100 bootstrap measures) (35) was conducted to characterize the relationship more accurately, because linear regression demonstrated a few data points with high leverages.

Results

Chemical composition of the experimental diets. Concentrates were of typical composition with respect to barley and molasses sugar beet pulp with differences in the oil composition only (Table 1). The grass silage was of good quality with a low pH, and had comparatively low ammonia-N · g^{-1} total-N with 30.1 g total-N · kg^{-1} DM (**Table 2**). More than 50% of the fatty acid existed in the form of 18:3(n-3) in the grass silage. LCPUFA such as 20:5(n-3), 22:5(n-3), and 22:6(n-3) were undetectable in F0. Supplementing the concentrate with fish oil increased the LCPUFA concentration roughly proportionally to the dietary concentration of fish oil (Table 2).

TABLE 2 Chemical composition and fatty acid profile of the experimental diets[1]

		Concentrate		
	Silage	F0	F1	F3
		g · kg^{-1} DM		
DM	249	872	873	876
Crude protein	188	161	164	163
NDF	500	274	271	286
Ammonia-N	2.89	N/A[2]	N/A[2]	N/A[2]
pH	3.72	N/A[2]	N/A[2]	N/A[2]
Lactic acid	123	N/A[2]	N/A[2]	N/A[2]
Fatty acid composition				
12:0	0.06	0.05	0.11	0.04
14:0	0.17	0.83	1.84	3.37
16:0	4.75	32.89	26.2	14.4
16:1	0.06	0.12	1.08	3.49
18:0	0.40	2.58	2.34	1.92
18:1(n-9)	0.53	22.42	18.94	12.11
18:2(n-6)	3.70	15.56	14.28	13.48
18:3(n-3)	16.29	1.29	1.42	1.93
20:0	0.12	0.20	0.19	0.17
20:5(n-3)	ND[3]	ND[3]	0.94	3.87
22:5(n-3)	ND[3]	ND[3]	0.25	1.00
22:6(n-3)	ND[3]	ND[3]	1.32	5.60
Total fatty acids	27.1	77.9	74.5	72.7

[1] Values are means; $n = 3$.
[2] N/A, Not applicable.
[3] ND, Not detected.

Nutrient intake, duodenal flow, and rumen fermentation characteristics. As indicated in the experimental design, DM intake was controlled, with the mean intake of 7.42 kg · d^{-1} with the targeted forage:concentrate ratio (60:40, DM basis) throughout the experiment. Duodenal flow of total-N indicated a net gain in steers fed diet F0 and F1 compared with the intakes and was not different between treatments (**Table 3**). Apparently digested NDF in the rumen ranged from 65 to 80% of intakes in all treatments. There were very few changes in rumen pH and the concentrations of ammonia-N and total VFA in the rumen (Table 3).

Fatty acids intake, duodenal flow, and biohydrogenation. Total fatty acid intake was different ($P < 0.001$) across treatments with steers fed F3 consuming 25 g · d^{-1} less than steers offered the F0 diets, although the difference was marginal (just 7% of F0) (**Table 4**). Duodenal flow of total fatty acids indicated a net gain of 52 and 73 g · d^{-1} in steers fed diets F0 and F1, respectively, but a net loss of 14 g · d^{-1} in steers fed diet F3 compared with the intakes. Increasing the concentration of fish oil culminated in greater intakes and flows of major LCPUFA, including 20:5(n-3), 22:5(n-3), and 22:6(n-3). Duodenal flow of 18:0 was much higher than intake in steers receiving all diets and decreased extensively with increasing concentration of fish oil from F0 to F3 ($P < 0.001$). Flow of total *trans* 18:1 increased 2.0-fold in steers fed diet F3 relative to steers fed diet F0 ($P < 0.001$) and this was largely due to increased *trans*-10 and *trans*-11, with these isomers contributing >50% of total *trans* 18:1 in digesta (**Table 5**). Further fractionation of total *cis* 18:1 showed that the *cis*-9 was largely responsible for the decrease in the duodenal flow of this fraction. The most commonly reported CLA isomers, namely *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, combined flow to the duodenum accounted for 52 and 50% of total CLA flow in steers fed the F1 and F3 diets, respectively (Table 5). Fish oil did not affect the duodenal flow of the *cis*-9, *trans*-11 isomer, while increasing fish oil had an elevatory effect on *trans*-10, *cis*-12 flow ($P < 0.001$). However, total CLA flow to the duodenum did not change, with *trans*-11 *trans*-13 most abundant in steers fed all diets. Biohydrogenation of 18:1(n-9), 18:2(n-6), and 18:3(n-3) ranged between 0.58 and 0.97 among treatments. Likewise, the majority of 20:5(n-3) and 22:6(n-3) was hydrogenated in the rumen and biohydrogenation of 22:5(n-3) was lower than the other LCPUFA (**Table 6**).

Microbial population profiling. PCR-DGGE analysis of 16S rRNA genes using primers universal for eubacteria indicated complex communities in all samples, with many similarities between steers receiving the F0 and F1 diets (**Fig. 1***A*). Diet F3 substantially altered the bacterial composition in the rumen of steers fed this diet. Cluster analysis separated steers fed diet F3 from those fed diets F0 and F1, with 77% of the band positions being the same (Fig. 1*A*). Band number also decreased ($P < 0.05$) in rumen samples taken from steers fed diet F3 compared to those fed diets F0 and F1 (**Table 7**). Similar analysis for members of the *Butyrivibrio* group showed a less complex pattern than eubacteria (Fig. 1*B*). Two main clusters were evident, separating

TABLE 3 Intake and duodenal flow of OM, total-N, NDF, and rumen fermentation parameters in steers receiving F0, F1, or F3 diets[1]

	Diets				Significance[2]	
	F0	F1	F3	SED	L	Q
Intake, $kg \cdot d^{-1}$						
OM	6.61	6.61	6.70	0.074	NS	NS
Total N	0.21	0.21	0.21	0.002	*	NS
NDF	3.08	2.92	3.10	0.034	NS	‡
Duodenal flow, $kg \cdot d^{-1}$						
OM	3.83	3.62	3.46	0.317	NS	NS
Total N	0.24	0.23	0.22	0.022	NS	NS
NDF	1.07	0.77	0.61	0.158	†	NS
Fermentation parameters						
pH	6.46	6.58	6.59	0.127	NS	NS
Ammonia-N, $mmol \cdot L^{-1}$	9.69	10.25	10.03	0.714	NS	NS
VFA (molar proportion)						
Acetate	58.9	57.8	57.1	0.81	*	NS
Propionate	22.6	22.9	23.3	0.66	NS	NS
Iso-Butyrate	1.40	1.46	1.45	0.127	NS	NS
n-Butyrate	13.2	13.5	14.1	0.665	NS	NS
Iso-Valerate	1.93	2.22	2.57	0.140	‡	NS
n-Valerate	1.73	1.70	1.70	0.117	NS	NS
Total VFA, $mmol \cdot L^{-1}$	78.9	77.7	79.8	4.81	NS	NS

[1] Values are means, $n = 6$.
[2] L, Linear response; Q, quadratic response; NS, not significant, $P \geq 0.05$; *$P < 0.1$; †$P < 0.05$; ‡$P < 0.01$.

TABLE 4 Fatty acid intake and duodenal flows in steers receiving F0, F1, or F3 diets[1]

	Diets				Significance[2]	
	F0	F1	F3	SED	L	Q
Intake	$g \cdot d^{-1}$					
12:0	0.45	4.07	2.94	0.108	§	§
14:0	2.92	5.80	10.13	0.157	§	†
16:0	116.2	94.0	57.6	1.29	§	*
18:0	9.23	8.28	6.81	0.091	§	NS
18:1(n-9)	66.3	54.7	33.3	0.75	§	NS
18:2(n-6)	59.4	56.9	54.3	0.60	§	NS
18:3(n-3)	78.4	78.8	81.0	0.94	†	NS
20:4	0.07	0.27	0.77	0.013	§	†
20:5(n-3)	ND[3]	2.87	10.65	0.169	§	N/A
22:5(n-3)	ND[3]	0.72	2.73	0.043	§	N/A
22:6(n-3)	ND[3]	3.88	15.29	0.241	§	N/A
Total fatty acids	344	331	319	3.6	§	NS
Duodenal flow						
12:0	0.58	1.08	0.50	0.092	§	§
14:0	2.93	4.67	4.70	0.275	§	§
16:0	118.8	112.2	65.3	9.50	§	NS
16:1	0.30	0.75	1.58	0.262	§	NS
18:0	152.7	115.1	58.9	19.09	§	NS
18:1 *trans* (total)	42.5	73.2	83.4	9.96	‡	*
18:1(n-9)	20.4	21.2	14.4	2.89	†	NS
18:2(n-6)	7.40	7.64	3.40	1.344	‡	NS
18:3(n-3)	3.32	3.71	2.08	0.734	*	NS
18:2 *cis*-9, *trans*-11	0.21	0.28	0.13	0.058	NS	*
20:4	0.34	0.45	0.24	0.127	NS	NS
20:5(n-3)	0.27	0.48	0.83	0.199	†	NS
22:5(n-3)	0.25	0.40	0.88	0.294	*	NS
22:6(n-3)	0.14	0.39	1.01	0.249	‡	NS
Total fatty acids	396	404	305	34.0	†	NS

[1] Values are means; $n = 6$.
[2] L, linear response; Q, quadratic response; N/A, not applicable; NS, not significant, $P \geq 0.05$; *$P < 0.1$; †$P < 0.05$; ‡$P < 0.01$; §$P < 0.001$.
[3] ND = not detected.

TABLE 5 Duodenal flow of 18:1 and CLA isomers in steers receiving F0, F1, or F3 diets[1]

	Diets				Significance[2]	
	F0	F1	F3	SEM	L	Q
Fatty acid flow	$g \cdot d^{-1}$					
18:1 *trans*						
4	0.38	0.35	0.17	0.017	§	†
5	0.16	0.21	0.18	0.024	NS	NS
6 + 7 + 8	2.74	4.13	3.64	0.641	NS	*
9	1.60	3.01	2.99	0.409	†	†
10	2.69	4.41	15.40	2.910	§	NS
11	19.1	40.4	40.9	6.705	†	†
12	3.01	5.72	6.00	0.515	§	‡
13 + 14 and *cis*-6	4.47	5.07	3.55	0.770	NS	NS
15 and *cis*-10	3.93	5.09	5.00	0.680	NS	NS
16	4.17	5.15	5.72	0.766	*	NS
Total 18:1 *trans*	42.5	73.2	83.4	9.96	‡	*
18:1 *cis*						
9	20.4	21.2	14.4	2.89	†	NS
11	0.98	1.72	2.78	0.408	§	NS
12	1.00	0.78	0.65	0.141	†	NS
13	0.04	0.58	0.75	0.222	†	NS
14	0	0.44	1.10	0.240	§	NS
15	1.02	1.07	2.02	0.201	§	NS
Total 18:1 *cis*	23.5	25.8	21.6	3.14	NS	NS
CLA[3]						
cis-9, *trans*-11	0.21	0.28	0.13	0.058	NS	*
trans-10, *cis*-12	0.09	0.26	0.49	0.070	§	NS
trans-11, *trans*-13 + *trans*-13, *trans*-15	0.62	0.44	0.54	0.094	NS	*
trans-7, *trans*-9 to *trans*-10, *trans*-12	0.06	0.07	0.05	0.033	NS	NS
Total CLA	0.96	1.03	1.23	0.180	NS	NS

[1] Values are means; $n = 6$.
[2] L, linear response; Q, quadratic response; N/A, not applicable; NS, not significant, $P \geq 0.05$; *$P < 0.1$; †$P < 0.05$; ‡$P < 0.01$; §$P < 0.001$.
[3] CLA isomers of double bond positions were identified according to Delmonte et al. (55).

steers fed diet F3 from those fed the other 2 diets, with 66% of the band positions being similar (Fig. 1*B*), and again band numbers were lower ($P < 0.05$) in F3 (Table 7). Subclusters between rumen samples derived from steers fed diets F0 and F1 were not clear in either analysis, indicating that supplementing fish oil at 1% of DM did not substantially alter microbial

TABLE 6 Biohydrogenation of unsaturated fatty acids in the rumen of steers receiving F0, F1, or F3 diets[1]

	Diets				Significance[2]	
	F0	F1	F3	SED	L	Q
	$g \cdot g^{-1}$					
18:1(n-9)	0.69	0.61	0.58	0.056	*	NS
18:2(n-6)	0.88	0.88	0.94	0.024	†	NS
18:3(n-3)	0.96	0.95	0.97	0.010	NS	NS
20:5(n-3)	N/A[2]	0.82	0.93	0.021	§	N/A
22:5(n-3)	N/A[2]	0.48	0.70	0.223	NS	N/A
22:6(n-3)	N/A[2]	0.90	0.94	0.031	NS	N/A

[1] Values are means; $n = 6$.
[2] L, linear response; Q, quadratic response; N/A, not applicable; NS, not significant, $P \geq 0.05$; *$P < 0.1$; †$P < 0.05$; §$P < 0.001$.

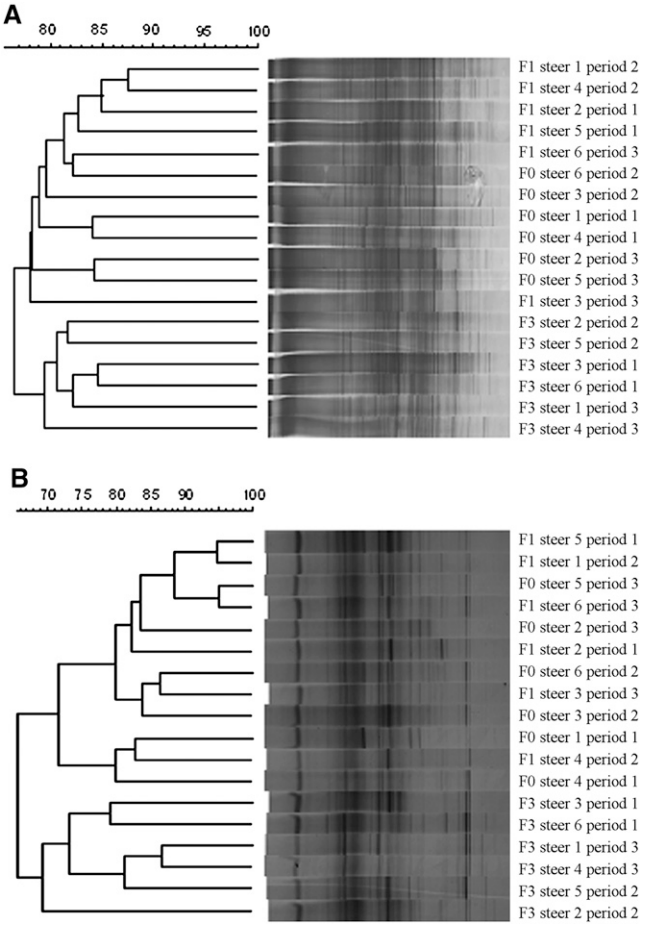

FIGURE 1 PCR-DGGE-derived unweighted pair group method with arithmetic mean dendrogram showing the effect of fish oil on the total eubacterial (*A*) and *Butyrivibrio* (*B*) populations in the rumen. F0, F1, and F3 relate to diets consisting of 0, 1, and 3% fish oil intake expressed in terms of DM intake. Scale relates to percent similarity.

population in the rumen. The Shannon-Weiner diversity index showed only minor differences in bacterial diversity for steers fed all diets with respect to both total eubacteria and the *Butyrivibrio* population (Table 7).

QPCR of 16S rRNA genes indicated that total eubacterial DNA concentration was not significantly different as fish oil increased (data not shown). The same was true of *C. proteoclasticum* 16S rRNA gene concentration (**Fig. 2***A*). When *C. proteoclasticum* numbers were expressed as a proportion of total eubacteria, there was no significant differences as fish oil increased (data not shown). A regression approach to the relationship between fish oil supplementation in the diets of the steers with the number of *C. proteoclasticum* in the rumen showed a significant relationship ($P < 0.05$), but the percentage variance accounted for (equivalent to adjusted r^2) was only 22.8, which was not sufficient to provide a reliable interpretation of the relationship (Fig. 2*B*). Therefore, bootstrap estimates from 100 bootstrap samples were performed to examine the correlation more accurately and the results were not fully supportive of a clear association between the 2 variables (Fig. 2*B*).

Discussion

Numerous reports have appeared in the literature demonstrating the effects of fish oil on ruminal biohydrogenation. The addition

TABLE 7 Band number and Shannon-Weiner diversity index calculated from the total eubacterial and the *Butyrivibrio* group-specific DGGE profiles obtained from rumen samples of steers receiving F0, F1, or F3 diets[1]

	Diets		
	F0	F1	F3
Total eubacteria			
Band number	69 ± 3.4[a]	66 ± 3.9[a]	58 ± 4.6[b]
Shannon-Weiner diversity index	6.56 (0.005)[a]	6.36 (0.007)[b]	6.61 (0.005)[b]
Butyrivibrio group specific			
Band number	69 ± 2.7[a]	67 ± 6.4[a]	61 ± 3.9[b]
Shannon-Weiner diversity index	4.37 (0.041)	4.18 (0.054)	4.51 (0.035)

[1] Values are means ± SEM, $n = 6$ or index (variance). Means in a row with superscripts without a common letter differ, $P < 0.05$.

of fish oil to ruminant rations has been used to manipulate LCPUFA concentrations of meat (2,4) and milk (36,37). Upon ingestion by ruminants, dietary lipid undergoes substantial lipolysis followed by microbial biohydrogenation to produce a high concentration of SFA in the rumen. One of the major pathways of biohydrogenation in the rumen is simplified as the conversion of free C18 PUFA to conjugated dienes or trienes, and then to 18:1 and finally by reduction to form 18:0 (11). The results of the present experiment were consistent with earlier findings in that the flow of 18:0 to the duodenum was significantly lower when fish oil was incorporated into the diet

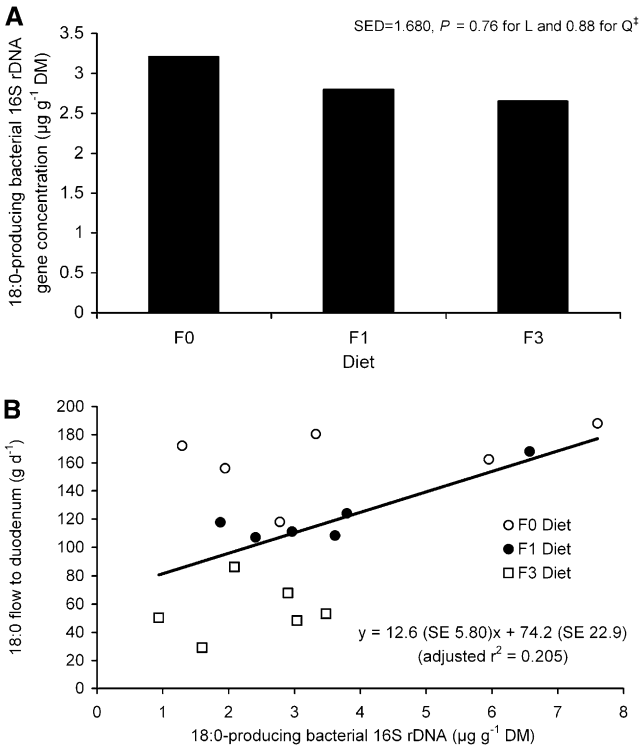

FIGURE 2 18:0-producing bacterial 16S rRNA gene concentration obtained from strained rumen fluid of steers given the experimental diets (*A*), and correlation between 18:0-producing bacterial 16S rRNA gene concentration and 18:0 flow to the duodenum (*B*). F0, F1, and F3 relate to diets consisting of 0, 1, and 3% fish oil intake expressed in terms of DM intake, $n = 6$. L, Linear response; Q, quadratic response.

of steers. The effect was also dependent on the concentration of the fish oil, as observed in other studies too (5,6,36). Several researchers reported a similar phenomenon with fish oil-supplemented diets (5–7,36) and suggested that the fish oil effect might involve an inhibition of the enzyme that catalyzes the final biohydrogenation step in the rumen. Alternatively, the proliferation of bacteria capable of conversion of 18:3 and 18:2 through to 18:0 might be inhibited by the LCPUFA in fish oil, resulting in increased *trans*-11 18:1. A recent study by Wąsowska et al. (38) showed that the addition of 20:5(n-3) or 22:6(n-3) (50 mg · L^{-1}) to pure cultures inhibited the growth and isomerase activity of *B. fibrisolvens*, whereas fish oil, in which the fatty acids are present as triacylglycerol, had no effect. Other reports (39,40) suggested that the accumulation of 18:2(n-6) in the rumen [by feeding more 18:2(n-6) to the ruminant] causes incomplete biohydrogenation. However, in the present study, the intake of 18:2(n-6) was similar with increasing concentrations of fish oil, excluding any possibility that 18:2(n-6) in the diets was responsible for the observed effect on biohydrogenation. Our data showed that fish oil at 3% of DM intake decreased the 18:0 flow and also increased the flow of *trans*-11 18:1 to the duodenum.

Increased flows of 20:5(n-3), 22:5(n-3), and 22:6(n-3) to the duodenum occurred in steers fed fish oil diets. The increased duodenal flow of these fatty acids in steers was lower than the increased dietary intake between diets F1 and F3. For example, the intake of 22:6(n-3) in steers fed diet F3 was 2.9-fold higher than that of steers fed diet F1 while only 1.6-fold higher in the duodenal flow. Biohydrogenation was presumably a major reason for this difference (5–7), although the degree to which these LCPUFA are biohydrogenated and the factors affecting biohydrogenation of LCPUFA in the rumen are still not well understood. Several studies have indicated that 20:5(n-3) and 22:6(n-3) are extensively metabolized in the rumen in vivo (5,6,41). In vitro studies have been less clear with some studies showing limited biohydrogenation (42), while others showed a decrease (43,44) or an increase in the extent of 20:5(n-3) and 22:6(n-3) biohydrogenation in relation to fish oil addition (45). It is also notable that although there was no intake of LCPUFA in the present study for the F0 diet, there were still measurable flows of these LCPUFA at the duodenum, which may reflect endogenous lipid from cell desquamation during digestive processes.

In terms of CLA, *cis*-9, *trans*-11 CLA was not the major isomer in duodenal digesta; *trans*-11, *trans*-13 CLA isomer accounted for ~50% of total CLA. Increased flow of *trans-trans* CLA leaving the rumen is consistent with previous reports of diets supplemented with fish oil (5,6,46). In addition, Lee et al. (7) reported that diets with high levels of 18:3(n-3), such as those based on forage or supplemented with oil rich in 18:3(n-3), produced *trans*-11, *trans*-13 as the predominant CLA isomer, possibly as a consequence of its involvement in the 18:3(n-3) biohydrogenation pathway. Duodenal flow of *trans*-10, *cis*-12 CLA, which is associated with modulating fat deposition (47) and milk fat depression (48), was significantly higher in steers fed the F3 diet. Several bacteria have been reported to convert 18:2(n-6) to *trans*-10, *cis*-12 CLA, including *Lactobacillus* spp. (49), *Propionibacterium acnes* (50), and *Megasphaera elsdenii* (51). However, to what extent these particular microorganisms play a role in ruminal biohydrogenation with the diet supplemented with fish oil remains unclear.

The microbial ecology of the rumen changed substantially with the addition of fish oil to the diet of steers, particularly at the higher inclusion rate. Many fewer bands were present in DGGE of 16S rRNA genes amplified by universal eubacterial primers. This reflects the toxicity of unsaturated fatty acids to

ruminal bacteria, which is particularly severe for cellulolytic species and also butyrate producers (52), although no difference in the concentration of ruminal butyrate was observed in the present study. The altered banding pattern presumably reflects the loss of the most sensitive species. Biohydrogenation occurs to detoxify the fatty acids. DGGE was also used to analyze the impact of fish oil on the *Butyrivibrio* population. These bacteria are believed to be the most active species involved in fatty acid biohydrogenation (39,53). Their sensitivity to PUFA is highly variable, with some members of the group sensitive to growth inhibition at PUFA concentrations of 5 mg/L (13) while others tolerate concentrations many times higher. The persistence of some bands but not others in the *Butyrivibrio* DGGE is consistent with this range of sensitivity.

Bacteria forming a small branch of the *Butyrivibrio* phylogenetic tree, clustering around *C. proteoclasticum*, are among the most sensitive ruminal species to the toxic effects of PUFA (13,15). They are also the only known ruminal species to convert *trans*-11 18:1 to 18:0 (10,15). *C. proteoclasticum* was originally isolated as a proteolytic species (54). Its name does not reflect its taxonomic position accurately, because it is not a spore former and is distantly related to true *Clostridium* species (15) and its name is in need of revision. Whether *C. proteoclasticum* truly represents the predominant 18:0 producers in the rumen is by no means certain and it is possible that other 18:0 producers have not yet been cultivated; indeed, some may not be cultivable at all using present culture techniques. If *C. proteoclasticum* is indeed the main 18:0 producer, a strong correlation might be expected between their numbers and the extent of biohydrogenation to 18:0 in digesta leaving the rumen. Primers and a probe designed to detect the *C. proteoclasticum* group and to exclude related *Butyrivibrio* that do not form 18:0 (34) were used in QPCR to assess the influence of fish oil on *C. proteoclasticum* numbers. The results were equivocal in the sense that a correlation was found, but it was rather weak, particularly when comparing different steers. It may be that other microbial species are involved, although no other ruminal bacteria, protozoa, or fungi are known to carry out the reaction to date. Alternatively, metabolic factors may be involved; for example, 18:0 formation occurs during the growth phase of *C. proteoclasticum*, not when it reaches stationary phase (15). Thus, the metabolic activity of *C. proteoclasticum* may not be proportional to 16S rRNA gene concentration and RNA may be a better marker.

In conclusion, these results are consistent with the hypothesis that fish oil has an inhibitory effect on the biohydrogenation of fatty acids in the rumen via its influence on microbial ecology. Total bacteria and *Butyrivibrio* populations were changed, consistent with the elimination of a number of species by fish oil. Numbers of *C. proteoclasticum* and duodenal fatty acid composition gave a weak correlation that neither offers strong support for nor against its predominant role in the process vis-à-vis other unknown bacteria that may convert *trans*-11 18:0 to 18:0. We are now attempting to link microbial changes to differences in fatty acid flow to the duodenum, using multivariate statistical approaches and sequencing of key bands, to identify other key bacteria that may be involved at various points of the biohydrogenation sequence. This information could potentially allow the development of novel strategies for manipulating this process, leading to the beneficial enhancement in nutritional value of ruminant products.

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Literature Cited

1. WHO. Diet, nutrition and prevention of chronic diseases. 2003. WHO Technical Report Series No. 916. p. 1–148.
2. Scollan N, Hocquette J-F, Nuernberg K, Dannenberger D, Richardson I, Moloney A. Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. Meat Sci. 2006;74:17–33.
3. Lock AL, Bauman DE. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids. 2004;39: 1197–206.
4. Scollan ND, Choi NJ, Kurt E, Fisher AV, Enser M, Wood JD. Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. Br J Nutr. 2001;85:115–24.
5. Shingfield KJ, Ahvenjarvi S, Toivonen V, Arola A, Nurmela KVV, Huhtanen P, Griinari JM. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. Anim Sci. 2003; 77:165–79.
6. Lee MRF, Tweed JKS, Moloney AP, Scollan ND. The effects of fish oil supplementation on rumen metabolism and the biohydrogenation of unsaturated fatty acids in beef steers given diets containing sunflower oil. Anim Sci. 2005;80:361–7.
7. Lee MRF, Shingfield KJ, Tweed JKS, Toivonen V, Huws SA, Scollan ND. Effect of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids in steers fed grass or red clover silages. Animal. In press 2008.
8. Piperova LS, Sampugna J, Teter BB, Kalscheur KF, Yurawecz MP, Ku Y, Morehouse KM, Erdman RA. Duodenal and milk trans octadecenoic acid and conjugated linoleic acid (CLA) isomers indicate that post-absorptive synthesis is the predominant source of cis-9-containing CLA in lactating dairy cows. J Nutr. 2002;132:1235–41.
9. Harfoot CG, Hazlewood GP. Lipid metabolism in the rumen. In: Hobson PN, Stewart CS, editors. The rumen microbial ecosystem. London: Chapman & Hall; 1997. p. 382–426.
10. Kemp P, White RW, Lander DJ. The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. J Gen Microbiol. 1975;90:100–14.
11. Kemp P, Lander DJ. Hydrogenation in vitro of alpha-linolenic acid to stearic acid by mixed cultures of pure strains of rumen bacteria. J Gen Microbiol. 1984;130:527–33.
12. Kopečný J, Zorec M, Mrázek J, Kobayashi Y, Marinšek-Logar R. *Butyrivibrio hungatei* sp nov and *Pseudobutyrivibrio xylanivorans* sp nov., butyrate-producing bacteria from the rumen. Int J Syst Evol Microbiol. 2003;53:201–9.
13. Paillard D, McKain N, Chaudhary LC, Walker ND, Pizette F, Koppova I, McEwan NR, Kopecny J, Vercoe PE, et al. Relation between phylogenetic position, lipid metabolism and butyrate production by different *Butyrivibrio*-like bacteria from the rumen. Antonie Van Leeuwenhoek. 2007;91:417–22.
14. van de Vossenberg J, Joblin KN. Biohydrogenation of C18 unsaturated fatty acids to stearic acid by a strain of *Butyrivibrio hungatei* from the bovine rumen. Lett Appl Microbiol. 2003;37:424–8.
15. Wallace RJ, Chaudhary LC, McKain N, McEwan NR, Richardson AJ, Vercoe PE, Walker ND, Paillard D. *Clostridium proteoclasticum*: a ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microbiol Lett. 2006;265:195–201.
16. Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR. Molecular ecological analysis of the gastrointestinal microbiota: a review. J Nutr. 2004;134:465–72.
17. Jarret IG. The production of rumen and abomasal fistulae in sheep. J Council Sci Ind Res. 1948;21:311–5.
18. Faichney GJ. The use of markers to partition digestion within the gastro-intestinal tract of ruminants. In: McDonald IW, Warner ACI, editors. Digestion and metabolism in the ruminant. Armidale (Australia): University of New England Publishing Unit; 1975. p. 277–91.
19. Lee MRF, Harris LJ, Dewhurst RJ, Merry RJ, Scollan ND. The effect of clover silages on long chain fatty acid rumen transformations and digestion in beef steers. Anim Sci. 2003;76:491–501.

20. Lee MRF, Harris LJ, Moorby JM, Humphreys MO, Theodorou MK, MacRae JC, Scollan ND. Rumen metabolism and nitrogen flow to the small intestine in steers offered *Lolium perenne* containing different levels of water-soluble carbohydrate. Anim Sci. 2002;74:587–96.
21. Sukhija PS, Palmquist DL. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J Agric Food Chem. 1988;36:1202–6.
22. Kramer JKG, Zhou JQ. Conjugated linoleic acid and octadecenoic acids: extraction and isolation of lipids. Eur J Lipid Sci Technol. 2001; 103:594–600.
23. Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J Bacteriol. 1996;178:5636–43.
24. Huws SA, Edwards JE, Kim EJ, Scollan ND. Specificity and sensitivity of eubacterial primers utilized for molecular profiling of bacteria within complex microbial ecosystems. J Microbiol Methods. 2007;70:565–9.
25. Klieve AV, Hennessy D, Ouwerkerk D, Forster RJ, Mackie RI, Attwood GT. Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. J Appl Microbiol. 2003;95:621–30.
26. Edwards JE, McEwan NR, Travis AJ, Wallace RJ. 16S rDNA library-based analysis of ruminal bacterial diversity. Antonie Van Leeuwenhoek. 2004;86:263–81.
27. Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM, Tiedje JM. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 2005;33:D294–6.
28. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.
29. Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, de Vos WM. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol. 2002; 68:114–23.
30. Sanguinetti CJ, Neto ED, Simpson AJG. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques. 1994;17:914–21.
31. Gafan GP, Lucas VS, Roberts GJ, Petrie A, Wilson M, Spratt DA. Statistical analyses of complex denaturing gradient gel electrophoresis profiles. J Clin Microbiol. 2005;43:3971–8.
32. Schlüter PM, Harris SA. Analysis of multilocus fingerprinting data sets containing missing data. Mol Ecol Notes. 2006;6:569–72.
33. Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, et al. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. FEMS Immunol Med Microbiol. 2003;39:81–6.
34. Paillard D, McKain N, Rincon MT, Shingfield KJ, Givens DI, Wallace RJ. Quantification of ruminal *Clostridium proteoclasticum* by real-time PCR using a molecular beacon approach. J Appl Microbiol. 2007;103:1251–61.
35. Payne RW, Murray DA, Harding SA, Baird DB, Soutar DM. GenStat for Windows. 9th ed. Introduction. Hemel Hempstead (UK): VSN International; 2006.
36. AbuGhazaleh AA, Schingoethe DJ, Hippen AR, Kalscheur KF, Whitlock LA. Fatty acid profiles of milk and rumen digesta from cows fed fish oil, extruded soybeans or their blend. J Dairy Sci. 2002;85:2266–76.
37. Ahnadi CE, Beswick N, Delbecchi L, Kennelly JJ, Lacasse P. Addition of fish oil to diets for dairy cows. II. Effects on milk fat and gene expression of mammary lipogenic enzymes. J Dairy Res. 2002;69:521–31.
38. Wąsowska I, Maia MRG, Niedźwiedzka KM, Czauderna M, Ribeiro J, Devillard E, Shingfield KJ, Wallace RJ. Influence of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids. Br J Nutr. 2006; 95:1199–211.
39. Polan CE, McNeill JJ, Tove SB. Biohydrogenation of unsaturated fatty acids by rumen bacteria. J Bacteriol. 1964;88:1056–64.
40. Harfoot CG, Noble RC, Moore JH. Factors influencing extent of biohydrogenation of linoleic acid by rumen microorganisms in vitro. J Sci Food Agric. 1973;24:961–70.
41. Scollan ND, Dhanoa MS, Choi NJ, Maeng WJ, Enser M, Wood JD. Biohydrogenation and digestion of long chain fatty acids in steers fed on different sources of lipid. J Agric Sci. 2001;136:345–55.
42. Chow TT, Fievez V, Moloney AP, Raes K, Demeyer D, De Smet S. Effect of fish oil on *in vitro* rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. Anim Feed Sci Technol. 2004;117:1–12.
43. Gulati SK, Ashes JR, Scott TW. Hydrogenation of eicosapentaenoic and docosahexaenoic acids and their incorporation into milk fat. Anim Feed Sci Technol. 1999;79:57–64.
44. Dohme F, Fievez V, Raes K, Demeyer DI. Increasing levels of two different fish oils lower ruminal biohydrogenation of eicosapentaenoic and docosahexaenoic acid in vitro. Anim Res. 2003;52:309–20.
45. AbuGhazaleh AA, Jenkins TC. Disappearance of docosahexaenoic and eicosapentaenoic acids from cultures of mixed ruminal microorganisms. J Dairy Sci. 2004;87:645–51.
46. Loor JJ, Ueda K, Ferlay A, Chilliard Y, Doreau M. Intestinal flow and digestibility of trans fatty acids and conjugated linoleic acids (CLA) in dairy cows fed a high-concentrate diet supplemented with fish oil, linseed oil, or sunflower oil. Anim Feed Sci Technol. 2005;119:203–25.
47. Park Y, Storkson J, Albright K, Liu W, Pariza M. Evidence that the *trans*-10,*cis*-12 isomer of conjugated linoleic acid induces body composition changes in mice. Lipids. 1999;34:235–41.
48. Shingfield KJ, Griinari JM. Role of biohydrogenation intermediates in milk fat depression. Eur J Lipid Sci Technol. 2007;109:799–816.
49. Alonso L, Cuesta EP, Gilliland SE. Production of free conjugated linoleic acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. J Dairy Sci. 2003;86:1941–6.
50. Liavonchanka A, Hornung E, Feussner I, Rudolph MG. Structure and mechanism of the *Propionibacterium acnes* polyunsaturated fatty acid isomerase. Proc Natl Acad Sci USA. 2006;103:2576–81.
51. Kim YJ, Liu RH, Rychlik JL, Russell JB. The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the trans-10, cis-12 isomer of conjugated linoleic acid. J Appl Microbiol. 2002;92:976–82.
52. Maia MRG, Chaudhary LC, Figueres L, Wallace RJ. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. Antonie Van Leeuwenhoek. 2007;91:303–14.
53. Jenkins TC, Wallace RJ, Moate PJ, Mosley EE. Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Anim Sci. 2008;86:397–412.
54. Attwood GT, Reilly K, Patel BKC. *Clostridium proteoclasticum* sp nov, a novel proteolytic bacterium from the bovine rumen. Int J Syst Bacteriol. 1996;46:753–8.
55. Delmonte P, Roach JAG, Mossoba MM, Losi G, Yurawecz MP. Synthesis, isolation, and GC analysis of all the 6,8-to 13,15-cis/trans conjugated linoleic acid isomers. Lipids. 2004;39:185–91.